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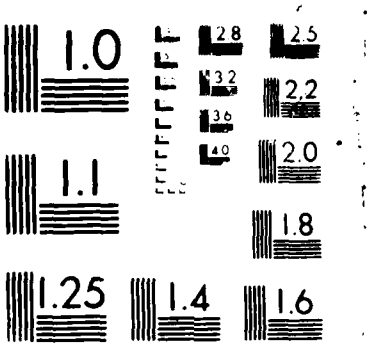
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DEVELOPMENT OF NOVEL, REVERSIBLE, NON-TOXIC ANTICOAGULANTS FOR GREATLY EXTENDED PLATELET STORAGE

ANNUAL REPORT

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June 19, 1987

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Summary

We are pleased to report that all facilities and resources outlined in the contract agreement are in place and the work is well underway. We have made considerable progress in the first phase of the research plan, namely the development and in vitro testing of novel anticoagulants for long-term liquid storage of platelets for transfusion. The first objective is to select reversible inhibitors of proteases or platelet activation that prove to be of benefit in maintenance of platelet viability. In order to save time and conserve resources, we have employed a series of "screening" experiments designed to eliminate compounds that have irreversible detrimental effects on platelet function. Only Thromboxane Synthetase inhibitors (like dazoxiben) remain to be tested.

Several new anticoagulants have been formulated following the screening tests. The best results were obtained with the combination of a thrombin inhibitor, a plasmin inhibitor, and inhibitors of platelet activation acting through cyclic AMP. The primary anticoagulant remains CPDA-1. Neither unfractionated nor low molecular weight heparin was suitable as a citrate substitute. Further improvements were achieved through manipulation of the bag surface-to-volume ratio.

We have begun investigation of the storage of platelets as platelet-rich plasma (PRP). The results indicate that platelets in PRP show signs of in vitro function and responsiveness even after storage for 15 days at 22°C. The new anticaogulant may extend the storage period even more. We believe that PRP may have great potential as a long-lasting forward resusciation fluid.

The focus of the second contract year will be on final selections of thrombin and plasmin inhibitors and on finding the minimal effective concentration of each component of the anticoagulant. In additon, the bag geometry will be optimized in both PRP and platelet concentrates to fit the lower metabolic rate permitted by the new anticoagulant. Presently, we can state that platelets stored 15 days with these new strategies are comparable by in vitro markers to standard platelet concentrates stored 7 days. We are hopeful that further advances are possible.

FOREWORD

For the protection of human subjects, the investigators have adhered to policies of applicable Federal Law 45CFR46.

Objectives and Results

The objective of the first two years of this study is to formulate a new anticoagulant that minimizes platelet activation and the resultant storage lesion. It has been shown in this work and elsewhere, that plasma proteases, especially thrombin and plasmin, can cause changes in platelets similar to those typifying the storage lesion. We have tested numerous protease inhibitors and several inhibitors of platelet activation in an attempt to screen out compounds with irreversible or toxic effects on platelet function.

The design of the experiments was as follows. A selected compound was delivered in 3.5 mL of vehicle (citrated saline) into a 25 mL aliquot of fresh citrated PRP ($n \geq 4$) in a small PL-146 transfer bag. Another aliquot of the same PRP preparation was treated with vehicle alone and both were stored 24 hours under standard conditions. The next day, samples were drawn for assay of pH, pCO_2, pO_2, platelet count, hypotonic shock recovery, and aggregation with 2×10^{-5} ADP. If significant differences were noted between the treated PRP and the control, then both were centrifuged and resuspended in autologous plasma free of inhibitors and assayed again after a 2 hour incubation at 37°C. Table 1 gives examples of compounds showing reversible inhibition of platelet function. In Table 2 is presented data for compounds showing irreversible effects on platelets.

Table 1. Screening tests of compounds showing reversible inhibition of platelet function (means, n=4).

	Plt. Count		Hypotonic Shock		Aggregation	
Agent	Pre	Post	Pre	Post	Pre	Post
PGE-1 + theophylline[1]	96%	80%	63%*	73%	0*	61%
Control	94%	70%	84%	68%	64%	54%
Forskolin	97%	83%	48%*	55%	0*	116%
Control	102%	73%	59%	59%	80%	128%
Calla bulb extract	96%	ND	42%*	54%	5%*	49%
Control	97%	ND	82%	59%	77%	66%

"Pre"= after 24 hour incubation but prior to removal of inhibitor.
"Post"= after resuspension and incubation in inhibitor-free plasma.
All results reported as percent of value obtained with fresh PRP.
*Significantly different from control, $p<0.05$.
[1]Five different doses tested, results from highest dose reported here.

Table 2. Screening tests of compounds showing irreversible effects on platelets (means, n≥4).

Agent	Plt. Count		Hypotonic Shock		Aggregation	
	Pre	Post	Pre	Post	Pre	Post
Fragmin[1]	64%	55%	83%	76%	73%	69%
Control	91%	73%	84%	91%	65%	79%

Unfractionated heparin - - platelets visibly clumped, testing impossible.

BABIM - - PRP clotted after 24 hour incubation.

Footnotes as in Table 1.

Many other compounds were tested and found to be without direct effect on platelet function. Below is given a brief summary of the results from preliminary storage studies using these agents singly or in combination.

Vitamin E (α-tocopherol): No obvious beneficial effect in PC (n=4).
Hydrocortisone: No obvious beneficial effect in PC (n=2).
5'AmidinoIndole: No obvious beneficial effect in PC (n=6).
FUT-175: Insoluble in plasma, precipitates with phosphate ions, no obvious beneficial effect in PC (n=8).
EACA: Slight beneficial effect in PC at high conc. (1 mM, n=8).
Pepstatin A: No obvious beneficial effect in PC (n=4).
Factor-deficient plasma: Infectivity risk to lab workers unacceptable.
Garlic (allicin): Beneficial effect in PC lost after 24 hours (n=4).
Leupeptin: Possible beneficial effect in PC, still under study.
Hirudin: Definite beneficial effect in PC, very expensive.
Thromstop: Definite beneficial effect in PC, less expensive alternative to hirudin, still under study.
Aprotinin: Definite beneficial effect in PC, still under study.
Thromboxane Synthetase inhibitors: To be tested.

Another compound of interest, DABE, is still unavailable to us. Apparently, the synthesis of DABE is extremely difficult. However, our success with other compounds from the lists above is not dependent on the availability of DABE.

In an attempt to limit the potential for bacterial growth in PC, we examined the effect of heparin and citrate based anticoagulant formulations including PGE-1 and theophylline on refrigerated platelets. Although all of the *in vitro* markers showed good preservation of platelet function for 10 days at 4°C, the platelets became irreversibly sphered, suggestive of poor transfusion quality. We have continually practiced careful arm scrub technique and discarded the first 10 mL of whole blood in order to limit bacterial contamination of PC. Every PC we study is examined at the end of the storage period by Gram stain. To date, none of our experimental PC has shown evidence of bacterial growth, and only a few of the controls have been positive.

New Anticoagulant

Our best results have been obtained with a formulation of:

CPDA-1 at 1:8 in whole blood plus
PGE-1 at 300 nM in whole blood plus
Theophylline at 1.9 mM plus
Aprotinin at 386 Kallikrein Inhibitor units/mL plus
Thromstop at 6 μM or
Hirudin at 4 Antithrombin units/mL

The best results with this new anticoagulant were obtained at a surface-to-volume (S/V) ratio of 4 cm^2/mL instead of the usual 7 cm^2/mL. We were able to take advantage of a lower surface-to-volume ratio because of the lower metabolic rate evident in PC prepared in the presence of these inhibitors. A comparison of S/V ratios in PC stored in the new anticoagulant (without a thrombin inhibitor) for 15 days is given in Table 3. The S/V ratio was controlled by folding and clamping the PL-732 storage container prior to delivery of a measured volume of PC. The storage data for PC prepared in the complete anticoagulant and stored at a S/V ratio of 4 cm2/mL are presented in Table 4. These data show that, by in vitro markers, the platelets stored 15 days in this anticoagulant were comparable to standardly citrated PC stored 7 days. Citrated PC stored 15 days at a S/V ratio of 4 cm^2/mL were as functionless as controls stored at 7 cm^2/mL.

Table 3. Effect of surface-to-volume ratio on PC stored 15 days in CPDA-1 containing PGE-1 + theophylline + aprotinin (means, n=4).

S/V Ratio	Plt. Count	pH	pCO_2	pO_2	Shock
2 cm^2/mL	clumped	5.84	7	187	0 %
4 "	61 %	6.58	18	135	81 %
7 "	31 %	6.61	9	180	19 %

Platelet count and hypotonic shock recovery rate are reported as percent of value in PC at Day 0.

Table 4. Comparison of PC stored 15 days in CPDA-1 + PGE-1 + theophylline + aprotinin + Thromstop or hirudin to standard citrated PC (n≥4).

		Count	pH	pCO_2	pO_2	Glucose	Shock	Agg-ADP	Discs
CPDA-1	D7	80 %	6.84	24	104	209	82 %	22 %	55 %
Hirudin +	D15	67 %	6.46	29	85	118	87 %	42 %	70 %
Thromstop +	D15	70 %	6.55	25	103	126	66 %	76 %	68 %
CPDA-1	D15	64 %	<6.0	6	170	15	0 %	0 %	0 %

Platelet count, aggregation rate (in response to 2×10^{-5} ADP, with platelets resuspended in inhibitor-free plasma) and hypotonic shock recovery rate are expressed as a percent of the value for fresh PC at Day 0. Glucose levels are reported as mg/dL remining in the PC plasma. Morphology is reported as percent of platelets appearing in discoid shape.

Storage of Platelet-rich Plasma

We have found that platelets stored as PRP instead of PC have better retention of *in vitro* responsiveness and viability during long-term storage experiments (see Table 5). The elimination of the second spin appears to avoid the initial activation of platelets and thus delay the onset of the storage lesion. Lowering the surface-to-volume ratio has been of benefit in the storage of PRP. We presume that this is permitted by a decreased metabolic rate relative to platelets in PC. Additional prolongation of the platelet shelf-life may be possible with supplementation of the citrate anticoagulant, as shown below with addition of PGE-1 and theophylline to CPDA-1. It may be possible to store platelets for 20 days with this methodology.

Table 5. Storage data for PRP at reduced S/V ratios (means, n=8).

		Count	pH	pCO_2	pO_2	Glucose	Shock	Agg-ADP
CPDA-1	D15	86 %	6.91	37	158	198	45 %	26 %
S/V=1.8	D20	75 %	6.64	23	178	140	7 %	14 %
PGE-1+Theo	D15	78 %	7.03	45	86	253	88 %	ND
S/V=1.8	D20	57 %	6.82	38	125	190	45 %	20 %
CPDA-1	D15	61 %	6.90	40	103	ND	60 %	39 %
S/V=1.3	D20	53 %	6.70	28	159	ND	25 %	16 %

Footnotes as in other tables.

Future Directions

Our continuing goal is to formulate a new anticoagulant strategy for preservation of platelet function and viability for at least 15 days. We have learned thus far that a combination of platelet activation inhibitors, a thrombin inhibitor, and a plasmin inhibitor work well together as supplements to CPDA-1. Further refinements may be necessary, such as use of a thromboxane synthetase inhibitor to reinforce the inhibitory activity of the cyclic AMP-active agents already employed. Inhibitors of calcium-activated proteases (ex., leupeptin) may also be of some benefit. However, the success achieved with the current formulation is not dependent on these refinements.

The surface-to-volume ratio currently used by blood banks in routine PC storage (approx. 7 cm^2/mL) has proved to be less than optimal in the storage of platelets in the new anticoagulant. There is a detrimental effect on stored platelets when the S/V ratio is too high, probably because of the rate of contact of platelets with the bag wall. Our preliminary data indicates that storage of either PC or PRP can benefit from reduced S/V ratios. We will continue these efforts to optimize the bag geometry for each preparation based on the metabolic demands of the platelets being stored.

We would like to suggest the consideration of two separate products for transfusion therapy: PRP and PC. It appears that platelets stored as PRP have the potential for greatly prolonged shelf-life with minimal chemical supplementation. PRP could serve as a forward resuscitation fluid for combat casualties who would not be compromised by the extra volume (relative to PC). PC would still be appropriate for post-op transfusion therapy.

Because of the very encouraging data generated thus far during 15 days of storage of PC or PRP in the new anticoagulant, we would like to start the survival studies ahead of the efficacy studies. We had originally planned to do efficacy studies in thrombocytopenic rabbits during Year 3 and then proceed to platelet survival studies in baboons in Year 4. One advantage in changing this schedule would be that we could assess the impact of the new anticoagulant formulations on platelet survivals over two years of testing (Years 3 and 4) and adjust components and concentrations accordingly. Another advantage would be the ability to study the effects of repeated transfusions of inhibitor-treated platelets in the baboons over a period of time that should reveal problems (if any) with antigenicity of the compounds in the anticoagulant. We would appreciate the reader's thoughts on this proposed change in work schedule.

In sum, we have produced an anticoagulant strategy that works well by *in vitro* assessment in the storage of platelets for 15 days. We plan to further refine the choice and concentration of reagents used in this strategy during the second year of this project. We hope that the USAMRDC is pleased with our progress.

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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The first phase of the development of a new anticoagulant formulation for extended storage of platelets is largely complete. Compounds which inhibit plasma proteases or platelet activation were screened for irreversible effects on platelets in short-term storage studies. Several combinations of reversible compounds were used as new anticoagulant formulations in long term storage studies which showed remarkable improvements in platelet viability by in vitro markers for up to 5 days at room temperature. The best results were obtained with the combined strategy of: elevation of cyclic AMP (PGE-1 plus theophylline), inhibition of plasmin and contact activation (aprotinin), and inhibition of thrombin (hirudin or thromstop). Platelets stored with these inhibitors showed signs of a lowered metabolic load and therefore could be contained in bags with a reduced surface-to-volume ratio. Further studies will be undertaken to finalize the formulation and bag geometry before proceeding to toxicity and efficacy studies in animal models.

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